REMARKS

This Supplement to Response is submitted as a supplement to the Response submitted on January 17, 2007 submitted in response to the Office Action of July 17, 2006. In the Response, Applicant submitted the Declaration of Dr. Karl K. Johe Under 37 C.F.R §1.132. The Declaration set forth Statements referring to certain attachments. Specifically, Statement 1 references the curriculum vitae of Karl K. Johe, and Statement 5 references a publication to Vescovi et al.. Accordingly, Applicants respectfully submit these attachments herewith.

If the Patent Office has any questions regarding this supplemental response, Applicants kindly request that the undersigned attorney of record be contacted directly to resolve any potential questions regarding same.

Respectfully submitted,

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Dated: January 18, 2007

Curriculum Vitae

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Awards:

Intramural Research Training Fellowship, NIH (10/93-1/97)
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Isolation and Cloning of Multipotential Stem Cells from the Embryonic Human CNS and Establishment of Transplantable Human Neural Stem Cell Lines by Epigenetic Stimulation

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Stem cells that can give rise to neurons, astroglia, and oligodendroglia have been found in the developing and adult central nervous system (CNS) of rodents. Yet, their existence within the human brain has not been documented, and the isolation and characterization of multipotent embryonic human neural stem cells have proven difficult to accomplish. We show that the developing human CNS embodies multipotent precursors that differ from their murine counterpart in that they require simultaneous, synergistic stimulation by both epidermal and fibroblast growth factor-2 to exhibit critical stem cell characteristics. Clonal analysis demonstrates that human C NS stem cells are multipotent and differentiate spontaneously into neurons, astrocytes, and oligodendrocytes when growth factors are removed. Subcloning and population analysis show their extensive self-renewal capacity and functional stability, their ability to maintain a steady growth profile, their multipotency, and a constant potential for neuronal differentiation for more than 2 years. The neurons generated by human stem cells over this period of time are electrophysiologically active. These cells are also cryopreservable. Finally, we demonstrate that the neuronal and glial progeny of long-term cultured human CNS stem cells can effectively survive transplantation into the lesioned striatum of adult rats. Tumor formation is not observed, even in immunodeficient hosts. Hence, as a consequence of their inherent biology, human CNS stem cells can establish stable, transplantable cell lines by epigenetic stimulation. These lines represent a renewable source of neurons and glia and may significantly facilitate research on human neurogenesis and the development of clinical neural transplantation. © 1999 Academic Press

Key Words: human CNS Stem Cells; transplantation; human neural precursors; EGF; FGF2.

INTRODUCTION

In the majority of tissues, specialized cells are known to originate from a small subset of highly undifferentiated, self-renewing elements which may persist throughout adulthood and are called "stem cells" (29). During development, stem cells generate intermediate progenitors whose differentiation and proliferation potential are progressively restricted until the generation of mature specialized cells is accomplished (23). As a consequence, embryonic tissues are characterized by the presence of an heterogeneous array of undifferentiated, mitotically active precursor cells possessing distinct developmental potential and alternative growth characteristics and requirements.

The central nervous system (CNS) represents no exception to this phenomenon. Thus, the unequivocal identification of a precursor cell as of a neural stem nature must be carried out on a functional basis, assessing its expression of the critical stem characteristics such as (i) undifferentiated features (as defined by the lack of differentiation markers), (ii) self-renewing capacity, (iii) pluripotentiality, and (iv) the ability to regenerate the tissue or elements of it (23, 29). Neural precursors that possess most of these defining characteristics and that qualify as "bona fide" stem cells have recently been found in the brain of rodents (1, 46, 55). Thus, self-renewing cells that have the capacity to give rise to neurons, and both glial cell types have been cultured from the developing (9, 20, 21, 34, 37, 50) and mature murine brain (14, 17, 20, 24, 36, 38, 48) by means of epigenetic stimulation. Their existence has also been confirmed by genetic means involving oncogene-mediated immortalization (see Ref. 56 for review).

To date, while progenitor cells have been isolated and cultured from the human CNS (30, 33, 41, 49), human neural stem cells exhibiting multipotentiality and capable of extensive self-renewal have not yet been

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identified, and the conditions developed for the culturing of rodent stem cells have proven ineffective in this system (8, 50).

Aside from answering the basic biological question of whether stem cells can be found in the developing human brain, their isolation and characterization have significant implications for the advancement of therapeutic approaches to neurodegenerative disorders (6, 15, 16). In fact, a major obstacle to the progression of therapies aimed at restoring neurological function by intracerebral transplantation of embryonic precursors is the source of donor material (4). In addition to the significant moral and ethical issues surrounding the procurement of human fetal tissue, other parameters such as age, storage, viability, and contamination must be standardized, making elective surgery difficult to schedule (31). To further compound the problem, multiple fetuses are usually required for a single transplant thereby introducing heterogeneity in the donor tissue and increasing the probability of immunological rejection or contamination (31).

Although an awareness of these difficulties has driven the search for alternative donor sources (10, 12, 26, 39, 40, 41, 47, 58), to date only two renewable sources of human neurons are available which have been established from teratocarcinoma (52) or from immortalized neural precursors (42). Since CNS stem cells have an extended self-renewal capacity and are multipotential, their isolation from human tissue would provide a renewable source of neurons, astrocytes, and oligodendrocytes, significantly reducing the need for fetal tissue in experimental and clinical neural transplantation (6, 13, 15).

We report the isolation of multipotential stem cells from the embryonic human brain, their extensive culturing to establish stable stem cell lines, and the successful engraftment of stem-cell-derived neuronal and glial progeny into the mature rodent brain.

RESULTS

Isolation, Expansion, and Multipotentiality of Human Neural Embryonic Stem Cells

We have established the conditions for isolating and propagating stem cells from various regions of the embryonic CNS and documented their multipotentiality by clonal analysis. Here, we report representative data for cells isolated from 10.5 weeks postconception (PCW) human diencephalon. Single cells from mechanically dissociated tissue were plated at 10^3 cells/cm^2 in the absence of growth factors (control) or in the presence of 20 ng/ml EGF or 10 ng/ml FGF2, either alone or in combination (see next paragraph for extensive description on the establishment of optimal growth conditions). No proliferation occurred in control medium and cells differentiated and subsequently died between 10 and 15 days *in vitro* (DIV). In cultures exposed to either EGF or FGF2, transient proliferation was observed followed by cell differentiation after 5–6 weeks and eventual death. Conversely, in the simultaneous presence of both GFs (growth medium), approximately 1% of the cells survived, began to divide, and gave rise to spherical clusters which were composed of undifferentiated neural precursors as demonstrated by the lack of any specific morphology, the absence of neuronal and glial antigens, and the expression of CNS precursor marker nestin (not shown). Cultures were serially passaged every 7–10 days by dissociation into a single cell suspension followed by replating in fresh growth medium. Their multipotentiality (i.e., their ability to generate neurons, astrocytes, and oligodendrocytes) was demonstrated by clonal analysis. Single stem cells (passages 20 through 34) were transferred by micromanipulation into a polyornithine-coated tissue culture well (1 cell/well) in growth medium, and a landmark was carved on the substrate to identify the field under analysis. Single stem cells were seen to proliferate and to give rise to spherical clones (Figs. 1A–1C), which were further subcloned to generate cultures that were differentiated in control medium (Fig. 1D). The progeny of a single cell were found to give rise to cells expressing neuronal, astroglial, and oligodendroglial antigens (Figs. 1E–1G). Quantitative immunocytochemistry showed that 10.1 ± 0.43, 74.5 ± 2.0, and 2.1 ± 0.06% of the total cells differentiated into neurons (β-tubulin), astrocytes (glial fibrillary acidic protein, GFAP), and oligodendrocytes (galactocerebroside, GC), respectively. Importantly, neuronal differentiation occurred gradually and neuronal processes elongated progressively over time. One, 2, and 3 days after inducing differentiation, 5.11 ± 0.42, 8.34 ± 0.32, and 10.53 ± 0.31% of the total cell number expressed the neuronal marker β-tubulin while the length of their main process increased from 36 ± 1.4 to 46 ± 1.7 and 55 ± 1.2 μm over the same time period (all data are from two experiments; means ± SE, $n = 10$; each increment in neurite length was statistically significant at $P < 0.05$).

Self-Renewal and Functional Stability of Human CNS Stem Cells: The Establishment of Neural Stem Cell Lines

We showed that the multipotent human precursors described above possess the ability to generate a large number of progeny and self-renewal capacity (23, 30). The ability of human CNS stem cells to generate a large number of progeny was demonstrated at the population level and was conditional on the establishment of an appropriate pattern of mitogenic stimulation by growth factors. In agreement with previous findings (8, 50), preliminary experiments showed that, when GFs were used alone, even saturating concentrations of either EGF or FGF2 would only induce transient cell division

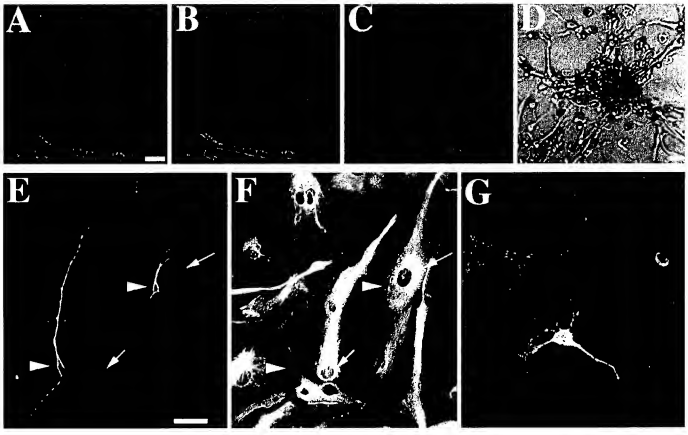

FIG. 1. Cloning of multipotential human CNS stem cells that generate neurons, astrocytes, and oligodendrocytes. A single cell from passage 22 stem cell cultures is shown 1 day after plating in growth medium; a landmark was carved on the plastic to identify the field (A). This cell proliferated (B, 11 DIV) and, by 24 DIV, gave rise to a spherical clone (C). Single clonal spheres were grown further and then subcultured to generate secondary and tertiary spheres. The progeny of a single cell was plated onto separate glass coverslips and allowed to expand (D). Differentiation was induced by removal of growth factors, and cultures were processed for immunofluorescence assay. Multiple immunolabeling shows the presence of neuronal (E, arrowheads, β-tubulin) and astroglial cells (F, arrows, GFAP) within a single sphere. Cells within another sphere from the same founder cell were identified as oligodendrocytes (G, GalC). Bars: A through C, 20 μm, bar in A; D, 60 μm; E through G, 35 μm, bar in E.

in embryonic human CNS cultures, with proliferation ceasing 5–6 weeks after plating (see also Fig. 2B). Hence, we investigated the effect of combining these two GFs. This was assessed by monitoring the [^{3}H]thymidine incorporation index in our cultures when the concentration of one of the two GFs was varied while maintaining the other at a fixed value. Twenty ng/ml of EGF and 10 ng/ml of FGF2 were the fixed concentrations chosen based on previous findings showing that these dosages warranted maximal proliferation in our system when each GF was used alone (data not shown). At concentrations of 20 ng/ml of EGF and 10 ng/ml of FGF2 used in combination, nearly maximal proliferation was achieved in our embryonic human CNS cultures without supersaturating the system. Using these optimal concentrations we found that human CNS stem cells can be exponentially expanded *in vitro* for more than 2 years (Figs. 2B and 2C), yielding an overall amplification of the total cell number of more than 10^5-fold (Fig. 2C, inset).

It is worth noting that self-renewal and cell expansion could also be obtained using concentrations lower than 20 ng/ml EGF and 10 ng/ml FGF2, with the lowest effective concentration being 5 ng/ml for each combined GF. However, as shown by the [^{3}H]thymidine incorporation experiments in Fig. 2A, the rate of cell expansion drops significantly at decreased GFs concentration, making the establishment of a continuous stem cell line more difficult.

Growing human CNS stem cells in the presence of different combinations of GF concentrations does not alter their capacity for neuronal differentiation. Stem cells grown in the presence of either 5 ng/ml of both EGF and FGF2, 20 ng/ml EGF and 10 ng/ml FGF2, or 100 ng/ml of both GFs gave rise to 11.46 ± 0.81, 10.53 ± 0.31, and 10.96 ± 0.45% neuronal cells over the total cell plated, respectively (data from two experiments; means ± SE, $n = 10$) following differentiation in GF-free medium.

Self-renewal was demonstrated by serial subcloning

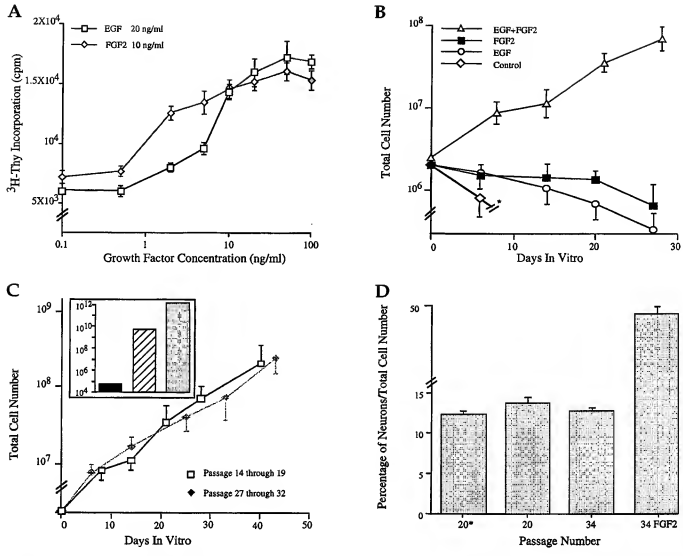

FIG. 2. Stable growth, expansion, and differentiation capacity of human embryonic CNS stem cells. (A) When used in combination, EGF and FGF2 significantly increase proliferation in human neural stem cell cultures. Dose–response curves were obtained by assessing [³H]thymidine incorporation when the concentration of one of the two GFs was varied between 0.1 and 100 ng/ml while maintaining the other constant. The constant concentrations chosen (20 ng/ml for EGF and 10 ng/ml for FGF2) were those that yielded maximal proliferation when each growth factor was used alone (not shown). The figure shows that maximal proliferation can be achieved when EGF and FGF2 are used together at 20 and 10 ng/ml, respectively (data are from a representative experiments; means ± SE, $n = 6$). (B) Human stem cells were subcultured under different conditions. In the simultaneous presence of both EGF and FGF2, the cultures continue to expand. When exposed to each of these growth factors alone, cells gradually decrease in number, differentiate after 4–5 weeks, and eventually die. In the absence of growth factors (control) growth ceases and death rapidly ensues (indicated by *). (C) Growth curves for cells from early (□, passages 14–19) and late cultures (◆, passages 27–32) displayed comparable growth rates. (Inset) Starting from 5×10^4 primary cells (■), 5×10^9 cells were generated and cryopreserved (▨); if all the cryopreserved cultures were expanded, more than 10^{12} cells would be available (▩). (D) Stem cells from early cultures (passage 20*) and from both early and late cultures (passages 20 and 34) that had undergone two cryopreservation cycles displayed equivalent neuronal differentiation capacity as they gave rise to comparable quantities of anti-β-tubulin-immunoreactive cells. Differentiation of stem cell cultures from late passages in the presence of low concentrations of FGF2 (0.5 ng/ml) results in a significant increase in neuronal differentiation; data from two experiments; means ± SE, $n = 10$. B and C, error bars are on a linear scale.

experiments where single clones (approximately 250 μm of diameter) were dissociated and replated in growth medium at less than 50 cells/cm² in a methylcellulose gel matrix to prevent cell aggregation. Cells from a single clone gave rise to an average of 12 ± 4 secondary spheres (diameter ranging from 150 to 300 μm; three experiments; means ± se; $n = 10$) that could either be differentiated into neurons and both types of glia or could generate tertiary clones (not shown). Notably, previous findings have shown that the genesis of terminally differentiated elements from neural stem cells may take place through the formation of interme-

diate neuronal or neuronal–astroglial progenitor cells (27, 51, 53). Thus, the fact our subcloning experiments did not demonstrate the presence of uni- or bipotential precursors may appear surprising. However, such oligopotential precursors are thought to belong to a transient dividing progenitor cell population and are likely endowed with limited proliferation capacity (23). Hence, since the reliable assessment of the full lineage potential of human stem cells required extensive expansion of a single cell progeny—so that analysis of distinct differentiated cell types could be carried out on multiple coverslips—uni- or bipotential progenitors are likely to have been eliminated during our protracted subcloning procedure. Due to the same phenomenon, these progenitor cells are likely to be routinely eliminated during long-term serial passaging in our cultures.

From a practical perspective, maintaining multipotent CNS stem cells in a self-renewing, expansive state by epigenetic stimulation allows us to establish continuous human neural stem cell lines. To support this, we showed the extensive functional stability of these cells over long-term culturing. For primary cultures, self-renewal was strictly dependent on the simultaneous, synergistic stimulation by EGF and FGF2 (Fig. 2B). When long-term cultures of human stem cells grown in the simultaneous presence of EGF and FGF2 were exposed to either growth factor alone, the total cell number gradually declined, and cultures completely ceased growth by 4–5 weeks *in vitro* (Fig. 2B). Similarly, when replated in control medium, cells rapidly ceased dividing, differentiated, and died (Fig. 2B). Of note, growth characteristics of human stem cells remained remarkably stable. Observation of single proliferating cells revealed that the first division always occurred after 3–4 DIV in cells from either early (10th) or late (34th) passages. Accordingly, the slope of the stem cell growth curve remained constant even after a significant number of serial passages (Fig. 2C). The differentiation capacity of human neural stem cells, and particularly their ability for generating neurons, remained unchanged over serial subculturing. When passage 20 and passage 34 cultures were differentiated, a comparable percentage of the total cells gave rise to neurons, measured by the β-tubulin immunoreactivity (Fig. 2D). It is worth noting that, similar to stem cells from the murine cortex (34), the fraction of stem-cell-generated neurons could be significantly increased by almost fourfold by allowing the stem cell progeny to differentiate in the presence of low concentrations of FGF2 (0.5 ng/ml; Fig. 2D).

Cryopreservation of human CNS stem cells was a reliable procedure with cell viability after thawing at between 70 and 95% of the total cell number. Indeed, the data described in this manuscript are from stem cell lines that had already undergone cryopreservation. Moreover, no change in the neuronal differentiation capacity of human CNS stem cells was observed following at least two freeze–thaw cycles (Fig. 2D).

Neuronal and glial characteristics of differentiated human CNS stem cell progeny were further assessed by routinely studying the expression of multiple lineage-specific markers in serially passaged cultures from passage 7 onward. Following growth factor removal, the neuronal antigens MAP2, MAP5, neuron-specific enolase (NSE), β-tubulin, Tau1, and neurofilaments (NFs) were detectable, together with astroglial (GFAP) and oligodendroglial (GC; myelin basic protein, MBP) antigens, by either immunocytochemistry (Figs. 3A–3I) or RT-PCR (Fig. 3J). Importantly, neurons prepared from long-term stem cell cultures were shown to express the ion channels necessary for excitability and were electrophysiologically active. In fact, neurons derived from passage 34 stem cells generate either single spikes in response to a brief current pulse (Fig. 3K, panel a) or firing when elicited by increasing depolarization (Fig. 3K, panels b–e). Voltage-clamp experiments indicated that a fast inward Na^+ current and two K^+ outward currents (a fast inactivating A-like and a delayed rectifier) could be recorded in these cells (not shown). Accordingly, the sustained firing (Fig. 3K, panel f) was completely blocked by 1 μm TTX (Fig. 3K, panel g), and after recovering (Fig. 3K, panel h) the application of 2 mM 4-AP (a blocker of A currents) resulted in the virtual disappearance of firing (Fig. 3K, panel i), which was fully restored after washing (Fig. 3K, panel l).

Finally, although space constraints have forced consideration of data for one representative (diencephalic) line, neural stem cell lines have also been established from various regions of 6- to 14.5-week-old human fetuses (Table 1).

Transplantation of Human CNS Stem Cells Progeny Into the Adult Rodent Brain

Future transplantation of human stem cell-derived neurons and glia will likely be conducted into the lesioned brain of human recipients whose immune system has been suppressed to minimize implant rejection. This is largely the case for intracerebral grafting of human embryonic tissue, the same kind of tissue from which human CNS stem cells are derived. Thus, while initial experiments indicated that transplantation of human stem cell-differentiated progeny could be effectively achieved in the intact brain of nonimmunosuppresed animals (data not shown), we have chosen to demonstrate the capacity of our cells to engraft following transplantation to the lesioned striatum of rats receiving cyclosporin daily.

Passage 30 to 34 stem cells were prelabeled with 5-bromodeoxyuridine (BrdU) while proliferating *in vitro;* the labeling index exceeded 98% of the total cells.

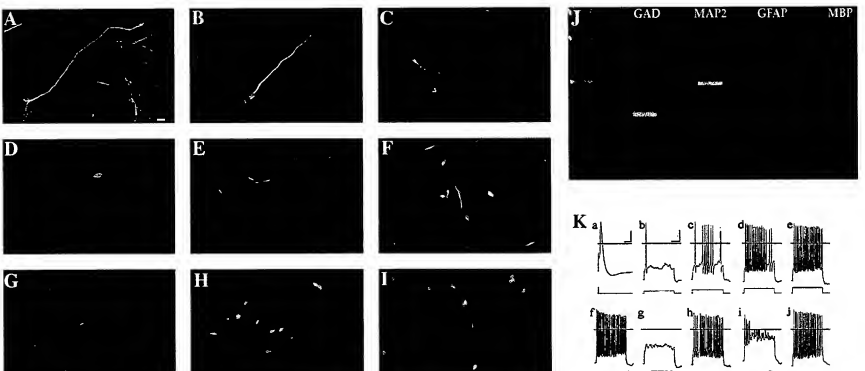

FIG. 3. Antigenic and functional characterization of human CNS stem cells differentiated progeny. From passage 7 onward, indirect immunolabeling and RT-PCR assays were used to further characterize the differentiated human stem cell progeny. The expression of multiple neuronal markers like MAP2 (A), MAP5 (B), Tau1 (C), NSE (D), NFs (E), and β-tubulin (F) together with positive immunoreactivity for GABA (H) and glutamate (I) and with the detection of mRNA for glutamic acid decarboxylase and MAP-2 (J) confirmed the neuronal differentiation capacity of human diencephalic stem cells. The detection of cells labeling with the A2B5 antibody (G) and the presence of mRNA for GFAP and for MBP (J) provided further evidence of the capacity for astroglial and oligodendroglial differentiation. Bars: A through E, 12 μm, bar in A; G, 6 μm; F, H, and I, 20 μm, bar in I. (K) Neurons generated from late-passage stem cell progeny (passage 34) were excitable. (a) A single action potential (peak up to +40 mV) elicited from resting potential (−52/−55 mV) by a brief (0.12 ms) current pulse (below); note the after-hyperpolarization and the well-defined threshold (−35 mV). (b–e) Responses to long-lasting depolarization (4 s) of increasing amplitudes (3, 4, 5, and 6 pA). Notice the increase in the firing frequency up to 30 Hz. (f–j) Firing (as in e) obtained before, during, and after the application of 1 μM TTX and 2 mM 4-amino-pyridine (4-AP). Vertical bars: 20 mV. Horizontal bars: a, 20 ms; b–j, 1 s. Horizontal lines, 0 mV.

TABLE 1

Representative List of the Stem Cell Lines Established from Various Regions of the Embryonic Human Brain at Various Developmental Ages

Brain region	Embryonic age	Embryo	Highest passage number	Average doubling time
Presumptive diencephalon	10.5 weeks	Normal	54	7–10 days
Presumptive diencephalon	10 weeks	Normal	34	12–15 days
Cortex	10 weeks	Normal	32	12–14 days
Cortex	6 weeks	Normal	30	10–12 days
Telencephalon	10.5 weeks	Normal	40	8–10 days
Lumbar spinal cord	12 weeks	Normal	32	15–20 days
Whole brain	9.5 weeks	Normal	26	7–10 days
Whole brain	14 weeks	Olivopontocerebellar atrophy (SCA1)	38	5–7 days
Whole brain	14.5 weeks	Fiederich's ataxia	36	5–7 days
Cortex	14 weeks	Down's syndrome	28	10–12 days
Brainstem	14 weeks	Down's syndrome	24	8–10 days
Lumbar spinal cord	14 weeks	Huntington disease	14	12–15 days

Following prelabeling, cells were differentiated for 6 days and the resulting neurons and glia were implanted into the ipsilateral striatum of 11 immunosuppresed adult rats lesioned unilaterally with 6-hydroxydopamine (3).

Surviving BrdU-immunoreactive cells (Figs. 4A and 4C) could be detected for up to 1 year posttransplantation. Quantitative analysis of four animals sacrificed 4.5 months following transplantation revealed that 12.3 ± 1.8% ($n = 6$) of the injected human cells had survived and some had migrated on average 1.2 mm rostrocaudally and 0.75 mm mediolaterally from the grafting site. Importantly, the cells detected by the anti-BrdU antibody coexpressed human specific ribonuclear proteins (Figs. 4A and 4B) or human mitochondrial markers (Figs. 4C and 4D), proving that viable transplanted human cells could be effectively detected by antibodies raised against specific human antigens. Using the anti-human ribonucleoprotein antibody (anti-human nuclei) in double labeling immunocytochemistry experiments, we were able to show that the transplanted cells can either differentiate into GFAP-immunoreactive astrocytes (Figs. 4E and 4F; high power magnification in Fig. 4I) or into β-tubulin-immunoreactive neurons (Figs. 4G and 4H; high power magnification in Fig. 4J). The neuronal nature of the transplanted human cells was further confirmed using an antibody raised against either human neurofilament (Fig. 4K) or human NSE (Fig. 4L). Due to the fibrous nature of both the anti-GFAP and anti-β-tubulin staining (Figs. 4F and 4H) a detailed assessment of the number of transplanted cells displaying astroglial and neuronal characteristics could not done. Nevertheless, it was clear that a significant number of these cells expressed the neuronal antigen β-tubulin (Figs. 4G and 4H), although a much higher fraction could be identified as astroglial cells (Figs. 4E and 4F). This finding was not unexpected since a high glial/neuronal ratio is typical of the stem cell-derived differentiated cell suspensions used for transplantation (see also Fig. 2D). No oligodendrocytes could be detected following transplantation. However, this was likely due to the fact that these cells represent a minimal fraction of the transplanted cells, so that their frequency *in vivo* could have fallen below the limit of detection. The issue of transplantation of stem cell-derived human oligodendrocyte thus requires further study.

Neural stem cell progeny were also implanted into the striatum of immunodeficient mice ($n = 6$). Up to 1 year after grafting, BrdU-immunoreactive cells could still be detected and the host parenchyma appeared histologically normal and tumor formation was not observed. These results demonstrate that the progeny of human CNS stem cell that have been extensively expanded *in vitro* can survive and migrate following transplantation into the adult CNS and can differentiate into mature neurons and glia.

DISCUSSION

In the absence of an identifying marker(s), neural stem cells are recognized based on the expression of defining functional features (reviewed in Ref. 23), which are not necessarily weighted equally in identifying a cell as stem. Essentially, the most critical stem cell characteristic is the ability to self-renew, which, by itself, identifies a stem cell (23, 29). The capacity to generate a wide array of differentiated functional cells and the potential to populate degenerating brain regions are also widely accepted as critical stem cell attributes. By these criteria, we describe here the initial isolation of multipotential stem cells from the embryonic human brain. We also report that human neural stem cells can establish continuous neural cell lines, thus providing a renewable source of differentiated neuronal and glial progeny that, similar to the

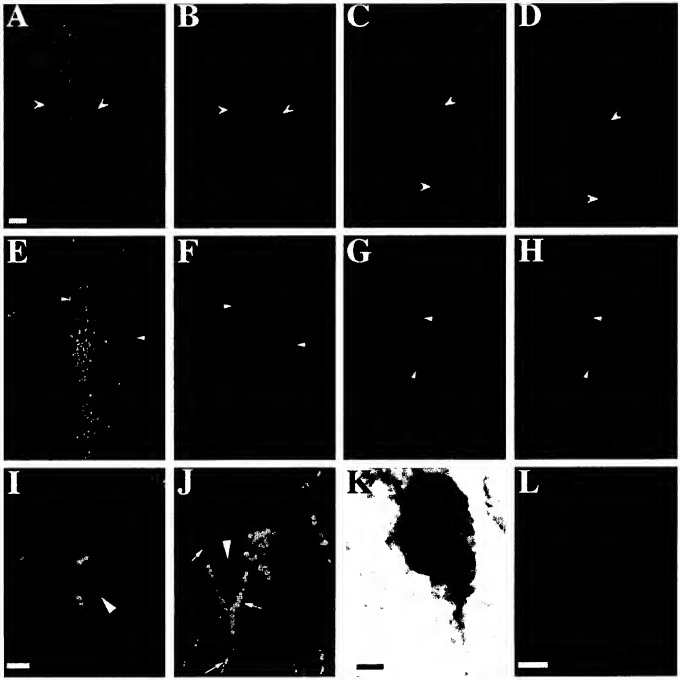

FIG. 4. Transplantation of CNS stem cell progeny into the adult rat striatum. Human diencephalic stem cells (passage 32) were prelabeled with BrdU (labeling index >98%) and allowed to spontaneously differentiate *in vitro* prior to transplantation into the ipsilateral striatum of adult rats unilaterally lesioned with 6-OHDA (6.5×10^4 cells/graft). Grafted cells were detected by double immunofluorescence assays for BrdU-positive cells (A, C) that label simultaneously (arrowheads) with an anti-human ribonuclear protein antibody (AHN, B) or with an anti-human mitochondria antibody (D). These assays showed that cells labeled with the AHN antibody (E, arrowheads) coexpressed the astroglial antigen GFAP (F, arrowheads); a high-power magnification of a double-labeled astrocyte is indicated in I by an arrowhead (AHN, green; GFAP, red). Similarly, AHN-immunoreactive cells coexpress the neuronal antigen β-tubulin (arrowheads in G, H; AHN, green; β-tubulin, red). A high-power magnification photograph of one of these neurons is shown in J, with the human-specific AHN antibody labeling the large nucleus of the cell (arrowhead, green) and the β-tubulin antigen confined in the scanty cytoplasmic area and in the neuronal processes (arrow, orange). The neuronal differentiation of transplanted cells is further demonstrated by detecting cells labeled with antibodies raised against human neurofilament (K) or human NSE (L). Bars: A through H, 100 μm, bar in A; I and J, 8 μm. bar in I; K, 5 μm; L, 15 μm.

fetal tissue from which stem cells derive, efficiently survive transplantation into the striatum of adult rodents.

Clonogenic and population analyses were used to demonstrate the stemness of the human precursors described here, leading to three major conclusions. First, not only do these cells possess self-renewal capacity—defined as the cell's ability to give rise to at least one daughter identical to itself at each cycle—but they undergo rounds of symmetric divisions in which both daughter cells possess stem characteristics. By this mechanism the stem cell population is steadily expanded at each generation in this system. Second, human neural stem cells are undifferentiated and multipotential, and are capable of giving rise to the three major differentiated neural lineages, neurons, astrocytes, and oligodendrocytes. Third, the synergistic stimulation by two growth factors, namely, EGF and FGF2, is necessary for the expression of these stem cell features, in culture. Thus, stemness may be regulated epigenetically in human neural precursors and requires the exposure of these cells to the appropriate combination of environmental cues to be brought about. Altogether, these observations emphasize the difference between human stem cells and their rodent counterpart which can self-renew when exposed to either of these growth factors alone (17, 36, 37). They also explain the apparent contradiction with previous reports describing the very limited proliferation potential of human precursors, although a direct comparison with our work is difficult as lineage potential and self-renewal capacity were not investigated in these studies (8, 50).

Stem cells have been described in various tissues such as skin, intestinal epithelium, and blood (23). However, despite the wealth of information available on these systems, consistent long-term proliferation of stem cells has not been achieved and cell lines for experimental studies are commonly established from various tissues from either tumors or through cell immortalization. This situation is exemplified in the field of human CNS precursors. To date, the only renewable source of human neurons is represented by immortalized oligopotential embryonic precursors (42) or teratocarcinoma-derived cells (52). We show here that epigenetic stimulation by growth factors allows for the establishment of lines of multipotential neural cells. In fact, we have exploited the cell expansion resulting from the self-renewal capacity of the human neural stem cells to significantly and consistently expand human CNS cells for longer than 2 years. Importantly, human CNS stem cell lines display remarkable functional stability, as their growth characteristics, dependence on growth factors, and potential for neuronal differentiation remain unchanged even after extensive subculturing. Furthermore, the expression of late neuronal antigens such as neurofilament proteins and the acquisition of distinct neuronal electrophysiological properties show that even the progeny of long-term cultured stem cells reach full neuronal maturation.

The finding that human CNS stem cell lines provide a renewable source of normal neurons, astrocytes, and oligodendrocytes suggests their application in lieu of fetal human tissue for neural transplantation. Hence, we have sought to demonstrate the capacity of CNS stem-cell-derived neuronal and glial progeny to engraft into the adult brain. In the most widely studied transplantation model, the lesioned striatum, human stem cell progeny showed an engraftment efficiency comparable to that of fetal tissue (31), the same kind of tissue from which they are derived. Furthermore, they exhibited substantial migration capacity, having been found dispersed as far as 1.2 mm from the graft in the host parenchyma. This characteristic may prove useful in a transplantation paradigm aiming at treating metabolic deficits in which repopulation of extensive areas of the brain parenchyma by transplanted cells may be of the essence (18, 44, 45). Although further experiments will be needed to fully assess the tumorigenic potential of human CNS stem cells, we never observed tumor formation, suggesting that the tumorigenic threat posed by these cell lines is minimal. Our observations are further supported by recent findings of the immortalization of human embryonic multipotential stem cells and their significant engrafting capacity into the postnatal rodent brain without any tumor formation ever being observed (Snyder *et al.*, unpublished observation).

Human stem cell lines may offer several less obvious advantages to experimental and clinical neural transplantation studies, as they provide the unprecedented opportunity to control some critical parameters in clinical neural transplantation (31). Cell differentiation can be initiated on one's own schedule and "fine-tuned" by modifying the culture medium. Hence, while cells can be transplanted as undifferentiated precursors whose differentiation would likely be directed by cues found in the host tissue (7, 22, 35, 43, 48), they may also be predifferentiated *in vitro* prior to grafting, thus allowing the experimenter to predetermine the type(s) of cell that will be delivered into the recipient's brain. Such variables as viability and cell composition could be monitored and manipulated and the characteristics of donor tissue may be "tailored" to fit specific graft applications. We showed the gradual maturation of stem-cell-derived neurons. Hence, donor neurons could be collected and grafted at predetermined stages of development to improve graft survival and integration (31). Our preliminary data also indicate that, as shown in rodent cells (20), the final outcome of the differentia-

tion that occurs can be altered so that almost half of the stem cell differentiated progeny acquires a neuronal fate (Fig. 2D). Alternatively, pure glial cultures for specific transplantation purposes (2, 5, 11, 19, 57) can be established from human stem cells by exploiting methodologies designed to generate glia from embryonic tissue (28). Finally, stem cell lines are now available as cryopreserved material, allowing for biosafety, sterility, and histocompatibility characterization far ahead of the day of surgery, which, in turn, could be electively scheduled.

Some CNS disorders may require grafting of specific neuronal subtypes. Since GABA appears to be the primary neurotransmitter phenotype of stem cell-derived neurons, these could be used to replace GABAergic spiny neurons in Huntington's disease (32). Recently we have been able to induce a significant fraction of the progeny of various stem cell lines to express catecholaminergic neuronal features (54) which may prove useful for cell replacement therapy in Parkinson's disease. Thus, in addition to markedly reducing the use of human fetal brain, stem cell lines may eventually eliminate the need for tissue exclusively from selected embryonic regions.

The availability of a renewable source of human neural tissue will likely find widespread application in various experimental or preclinical fields. Since CNS precursors can be transduced with specific genes (39, 41, 44, 45, 52) human CNS stem cell lines could serve as a cellular platform for gene therapy in the CNS. Alternatively, proper tissue culture models could be established for basic studies on human neurogenesis or for high-throughput screening procedures for CNS drug discovery. Additionally, novel genes, relevant to human CNS development, could be isolated by applying differential display or library subtraction techniques to cultures of human CNS stem cells at progressive stages of differentiation. The establishment of CNS stem cell lines from human fetuses carrying genetic mutations responsible for major human brain pathologies such as olivopontocerebellar atrophy, Friederich's ataxia, and amyloidosis in Down's syndrome (Table I) may serve to set up *ex vivo* models which are presently unavailable for dissecting the mechanisms underlying specific brain diseases.

METHODS

Establishment of Human CNS Stem Cell Lines

Permission to use human CNS tissue was obtained by the ethical committee of the "Neurological Institute C. Besta" and of the Obstetric–Gynecological Clinic (ICP, Milan, Italy). Tissue procurement is in agreement with the ethical guidelines of the European Network for Transplantation (NECTAR). Tissue was obtained after 6 to 12 weeks of gestation from routine legal abortion (15 weeks for therapeutic abortion) and mechanically triturated. Single cells were plated at 1000 cells/cm^2 in untreated 25-cm^2 tissue culture flasks (Nunc, USA), in the presence of 20 and 10 ng/ml of human recombinant EGF and FGF2, respectively, in NS-A basal serum-free medium (Euroclone, Irvine, Scotland) containing 2 mM L-glutamine, 0.6% glucose, 9.6 μg/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin, 0.1 mg/ml transferrin (sodium salt, grade II; Sigma; control medium).

After 4 weeks, cultures were harvested, mechanically dissociated, and replated under the same conditions. After performing this procedure twice to eliminate short-term dividing precursors, bulk cultures were generated by passaging cells at higher density (10^4 cells/cm^2) every 7–10 days in the same growth medium. Cells were counted and viability was determined at every passage by trypan blue exclusion. For clonal analysis see Fig. 1. For differentiation studies, stem cell progeny were plated onto polyornithine-coated coverslips in DMEM/F12 serum-free medium containing the same concentrations of putrescine, progesterone, apotransferrin, sodium selenite, and insulin reported above and subjected to specific assays at the appropriate time. Samples for RT-PCR assay were prepared from bulk cultures grown on polyornithine-coated dishes.

[^{3}H]Thymidine Incorporation Assay

Cells were washed twice with control medium and plated into polyornithine-coated 96-well plates (1.5×10^4 cells/well) in the presence of the GFs. Four days after plating, cells were exposed to 1 μCi/well of [*methyl*-^{3}H]thymidine (74 GBq/mmol, Amersham) for 48 h. Cells were lysed using distilled water and DNA was blotted onto Titerteck Filter Paper (ICN) using a Cell Harvester (Flow Laboratories). Following thorough rinsing, filters were transferred into 4 ml of scintillation medium (Beckman) and counted using a β-scintillation counter (Wallac).

Cryopreservation

Freezing. Six to 8 days after the last subculturing step, cells were harvested by gentle pipetting and centrifuged at 800*g*. The pellet was washed once with fresh growth medium and cells were resuspended in the same medium containing 10% DMSO (freezing medium) to yield a final concentration of 1.5×10^6 cell/ml. Cells were transferred into 2-ml cryogenic vials (Nalgene) and, following an equilibration phase of 15 min at room temperature, cells were gradually cooled at a rate of 1°C/min and eventually stored in liquid nitrogen.

Thawing. Vials were quickly thawed by immersion in a 37°C water bath and cells were gently resuspended in 12 ml of growth medium and rinsed twice with the same medium prior to replating under standard growth conditions.

Electrophysiological Analysis

Stem cell progeny were allowed to differentiate spontaneously for 21DIV as described above. Patch-clamp experiments were performed as previously described (17). Voltage-clamp currents and current-clamp recordings were obtained with an Axoclamp 200A or a home-designed amplifier (25). Recordings were performed at 21–23°C and coverslips were replaced after 30–45 min.

Immunocytochemistry

Immunostaining was performed as described elsewhere (17). Briefly, cells were fixed for 20 min in 4% paraformahaldeyde in PBS, pH 7.4, washed, and incubated for 90 min at 37°C with PBS/0.1% Triton X containing 10% normal goat serum and the appropriate antibodies (see below). Following washing, cultures were incubated for 45 min at room temperature with the secondary antibodies, washed, counterstained with DAPI, and mounted using Fluorsave. Immunolabeling of transplanted cells was performed on serial brain sections. Animals were anesthetized with sodium pentobarbital (intraperitoneal injection of Nembutal, 0.1 ml) and perfused with 10% formalin; brains were removed and postfixed for 24 h and then cryoprotected in 10 and 20% sucrose in PBS overnight at 4°C, followed by an overnight embedding step in a 2:1 (v/v) 20% sucrose/Tissue-Tek OCT embedding compound mixture. Ten-micrometer-thick serial sections were cut in a cryostat and mounted onto gelatin-coated glass slides for double immunolabeling. Briefly, sections were air-dried and rinsed in 0.1 M Tris–HCl buffer (pH 7.6) containing 0.005% BSA and 0.1% Triton and incubated with the primary antibody for 24–72 h at 4°C. After thorough rinsing, sections were incubated for 2 h at room with the appropriate secondary fluorescein-conjugated antibody, rinsed again, and incubated with the second primary antibody as above. Following further rinsing and incubation with a CY3-conjugated secondary antibody, sections were washed and coverslipped with Fluorsave. For BrdU detection in double-immunolabeling procedures, sections were incubated in 1.0 N HCl for 30 min at 60°C and rinsed three times in PBS prior to double-immunolabelling with anti-BrdU and either anti-human nuclei or anti-human mitochondria antibodies. All samples were viewed under a Zeiss Axiophot-2 microscope. Proper controls for primary and secondary antibodies revealed neither nonspecific staining nor antibody cross-reactivity.

	Primary antibodies	
NSE, GFAP	Rabbit polyclonal; ready to use	Incstar, U.S.A.
MAP2, MAP5, Tau1	Mouse monoclonal; 1:200	Boheringer Mannheim, Germany
GalC	Mouse monoclonal; 1:50	Boheringer Mannheim, Germany
β-Tubulin,	Mouse monoclonal; 1:1000	Sigma, U.S.A.
GABA	Rabbit antiserum; 1:5000	Sigma, U.S.A.
Neurofilaments Mix (SM 311)	Mouse monoclonal 1:1000	Sternberger, U.S.A.
Anti-human neurofilament	Mouse monoclonal 1:100	Boheringer Mannheim, Germany
Anti-human mitochondria	Mouse monoclonal 1:20	Chemicon, U.S.A.
Anti-human nuclei	Mouse monoclonal 1:20	Chemicon, U.S.A.
Anti-human NSE	Rabbit polyclonal 1:100	Polysciences, U.S.A.
Anti-human neurofilament (160kD)	Mouse monoclonal 1:50	Boheringer Mannheim, Germany
Anti-BrdU	Rat polyclonal 1:50	Seralab

Rabbit antiserum to glutamate (1:20,000); a gift from Dr. Petruz, Univ. of North Carolina, through Dr. R. Spreafico.

RT-PCR

Cells were collected by centrifugation and total RNA was isolated using Trizol according to manufacturer's protocol (Gibco-BRL). Total RNA was reverse transcribed into cDNA with Superscript II RnaseH$^-$ reverse transcriptase using oligo $(dT)_{12-18}$ (Pharmacia) as primer, according to manufacturer's instructions. PCR primers were designed using PrimerDesigner (Scientific Educational Software). The PCR reaction was carried out in a 25-μl volume containing 1 unit of *Taq* polymerase (Gibco-BRL), 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 50 pmol of specific primer, 5 nmol of the four nucleotides, and RT product equivalent to 20 ng RNA. Thermal profile: 4 min at 94°C, followed by 40 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min, and 7 min at 72°C. The PCR products were separated on a 2% agarose gel at 10 V/cm and visualized by ethidium bromide.

Primers

GFAP 5′ - TCA TCG CTC AGG AGG TCC TT - 3′
5′ - CTG TTG CCA GAG ATG GAG GTT - 3′
Expected product size: 383 bp

MAP2 5′ - GAA GAC TCG CAT CCG AAT GG - 3′
5′ - CGC AGG ATA GGA GGA AGA GAC T - 3′
Expected product size: 527 bp

MBP 5′ - TTA GCT GAA TTC GCG TGT GG - 3′
5′ - GAG GAA GTG AAT GAG CCG GTT A - 3′
Expected product size: 379 bp

GAD 5′ - GCG CCA TAT CCA ACA GTG ACA G - 3′
5′ - GCC AGC AGT TGC ATT GAC ATA A - 3′
Expected product size: 284 bp

Transplantation

Wistar rats (200–225 g) received intranigral infusion of 6-OHDA and animals bearing effective lesion were selected as graft recipients as described previously (3). Cells were trypsinized at 37°C for 2 min and mechanically dissociated to a single cell suspension. One to 3 μl

of a 2.5×10^7 cell/ml suspension were transplanted 2 weeks postlesion via a 30-gauge cannula at a controlled rate of 0.1–0.5 μl/min. Stereotaxic coordinates: A/P, +0.5; M/L, −3.1; D/V, −4.8. Rats received CyclosporinA (Sandimmune; 0.1 mg/ml) with the drinking water for the duration of the posttransplantation period.

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